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Microautophagy and macropexophagy may occur simultaneously in *Hansenula polymorpha*

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Abstract We subjected methanol-grown cells of wild type *Hansenula polymorpha* simultaneously to nitrogen depletion and excess glucose conditions. Both treatments induce the degradation of peroxisomes, either selective (via excess glucose) or non-selective (via nitrogen limitation). Our combined data strongly suggest that both processes occur simultaneously under these conditions. The implications of these findings on studies of autophagy and related transport pathways to the vacuole in yeast are discussed.

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Keywords: Methylophilic yeast; Pexophagy; Methanol; Protein degradation; Vacuole

1. Introduction

In eukaryotic cells, organelle functions are specified by their unique sets of resident proteins. Maintenance of organelle integrity, structure and function requires effective regulatory mechanisms to balance biosynthetic and inactivation activities. The ability to degrade and reuse cellular components is instrumental to maintain cellular homeostasis [1–3]. Yeast peroxisomes are favorable models for studies of organelle homeostasis because both the proliferation and degradation of these organelles can be readily manipulated [4].

Peroxisome degradation in the methylotrophic yeast *Hansenula polymorpha* may occur via two morphologically distinct machineries, namely microautophagy and macropexophagy (see [4]). At nitrogen-limiting conditions portions of the cytoplasm – also including organelles like mitochondria and peroxisomes – are delivered via a phagocytosis-like mechanism to the vacuole for degradation in a non-selective way, a process called microautophagy [4,5]. The second mode – macropexophagy – is induced at methanol-utilization repressing conditions (e.g., after a shift from methanol to glucose or ethanol) and involves the selective and sequential degradation of individual peroxisomes via sequestration and subsequent fusion of the sequestered compartments with the vacuole [6,7]. Earlier studies have indicated that many protein transport pathways to the vacuole, including microautophagy and macropexophagy, partly overlap [8–11].

The mechanism by which the nutritional signal is transduced into a specific machinery of organelle degradation is not completely known. The mode of signaling strongly determines the ultimate way by which the cargo is delivered to the vacuole. As stated above, in *H. polymorpha* excess glucose/ethanol conditions initiate peroxisome degradation via macropexophagy, while nitrogen depletion results in microautophagy. However, species related differences appear to exist. In *Pichia pastoris*, for instance, ethanol and glucose bring about different modes of peroxisome degradation namely either macropexophagy (induced by ethanol) or a process morphologically similar to microautophagy in which the vacuole engulfs clusters of peroxisomes, designated micropexophagy (induced by glucose) [12–14].

In baker's yeast, however, peroxisome degradation has generally been studied by applying excess glucose and nitrogen limitation conditions simultaneously [15–17]. This implies that in this yeast species organelle degradation may result from either macropexophagy or microautophagy, while it remains unclear which one is in fact operative at any given time point. To shed more light on this fundamental question, we investigated the response of methanol-grown *H. polymorpha* cells simultaneously exposed to excess glucose and nitrogen limitation conditions. *H. polymorpha* is a model organism of choice for such studies, since the morphological events accompanying these two autophagy-related processes are much more pronounced relative to baker's yeast.

2. Materials and methods

2.1. Micro-organisms and growth conditions

Wild-type *Hansenula polymorpha* CBS4732 cells (Centraal Bureau voor Schimmel cultures (CBS) culture collection, Utrecht, The Netherlands) were grown in batch cultures on mineral media (MM) [18] using glucose (0.5% w/v) or methanol (0.5% v/v) as carbon source and 0.25% ammonium sulfate as nitrogen source. In microautophagy experiments, cells were grown in MM supplemented with methanol and ammonium sulfate and subsequently shifted to MM supplemented with methanol but lacking yeast extract and ammonium sulfate [5]. Macropexophagy was induced by adding 0.5% glucose to methanol-grown cells [19]. Samples were taken at various time points for biochemical and ultra structural analysis.

For biochemistry equal volumes of the cultures were taken per sample in each individual experiment in order to correct for dilution of proteins due to growth. Samples were TCA precipitated and stored at –20 °C. After preparation of crude extracts, equal volumes of extract were loaded per lane.

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2.2. Biochemical methods

Crude cell extracts were prepared as described elsewhere [20]. SDS-PAGE and Western blotting were performed by established procedures [21].

2.3. Morphological analysis

Intact cells were prepared for electron microscopy and immunocytochemistry as described elsewhere [22]. The average number of peroxisomes per cell was estimated by random counting of cell profiles in thin sections of KMnO_4 -fixed cells [23].

3. Results

3.1. Simultaneous exposure of *H. polymorpha* cells to excess glucose and nitrogen limitation conditions induces enhanced peroxisome degradation rates

Wild-type *H. polymorpha* CBS4732 cells were grown in methanol/ammonium sulfate-containing media until the mid-exponential growth stage ($\text{OD}_{660} = 1.5$) and subsequently shifted to the following fresh environments: (i) methanol in the absence of nitrogen, (ii) methanol/ammonium sulfate supplemented with excess glucose (0.5% final concentration) and (iii) methanol lacking nitrogen but supplemented with excess glucose (0.5%) at the same time. Samples were taken at different time points and prepared for Western blot analysis. Western blots were decorated with specific antibodies against the peroxisomal matrix protein alcohol oxidase (AO). The results (Fig. 1) indicate that simultaneous adaptation of the cells to excess glucose and nitrogen-limitation conditions resulted in a significantly stronger reduction of AO protein levels, relative to conditions of excess glucose or nitrogen limitation alone.

3.2. Microautophagy and macropexophagy occur simultaneously in *H. polymorpha* when both processes are induced at the same time

Since the morphological events that accompany microautophagy or macropexophagy in *H. polymorpha* differ strongly, electron microscopy was used to determine the morphology and the kinetics of both processes in cells under conditions that these were induced simultaneously.

Inspection of ultrathin sections of KMnO_4 -fixed cells revealed that in methanol/ammonium sulfate-grown *H. polymorpha* WT cells, exposed to excess glucose, individual peroxisomes became sequestered from the cytosol by multiple membrane layers, as described previously [6,7]. Sequestration

was already detectable after 15 min of incubation of cells in the presence of glucose (Fig. 2A). Organelle degradation, demonstrated by the accumulation of peroxisomal AO into vacuoles by immunocytochemistry, was evident already after 30 min of incubation (Fig. 2F). Additionally, exposure of cells to nitrogen-limiting conditions showed the expected morphology of organelle degradation (data not shown; [5]). Detailed experiments on cells subjected to both nitrogen limitation and glucose excess conditions, also including analysis of serial sections, demonstrated the characteristic morphological events of both macropexophagy and microautophagy occurring simultaneously. Thus, both specific organelle sequestration and the typical phagocytosis-like characteristics of non-specific cytoplasm uptake via microautophagy were frequently detectable in one and the same cell (Fig. 2B). Apparently, initiation of macropexophagy does not exclude degradation of organelles by microautophagy. Furthermore, occasionally cells have been observed in which organelles that were in the process of being sequestered were also subject to microautophagy (Fig. 2C and D). As a result, apparently intact peroxisomes were regularly observed in autophagic vacuoles (Fig. 2E and F). These findings are indicative for the novel view that tagging of organelles for macropexophagy apparently does not exclude them from the other degradation machinery. It should be noted that organelle sequestration was never observed in cells subjected to nitrogen limitation alone. Conversely, phagocytosis-like uptake of organelles in the vacuole was not observed in cells exposed to excess glucose.

To seek further quantitative evidence for the above finding, morphological analyses were performed to analyze the kinetics of peroxisome destruction at the three conditions (macropexophagy and microautophagy alone, and the two processes simultaneously). As is evident from the data, summarized in Fig. 3, already after 1 h of exposure to both conditions simultaneously, the number of peroxisomes was drastically reduced relative to each single condition separately. These data strongly suggest that microautophagy and macropexophagy occurred at the same time in cells simultaneously exposed to both nitrogen limitation and glucose excess conditions, thus leading to enhanced uptake of peroxisomes in the vacuole.

4. Discussion

We have shown that in *H. polymorpha* two modes of peroxisome degradation, namely glucose-induced selective peroxisome degradation (macropexophagy) and nitrogen limitation-induced non-selective degradation (microautophagy), can occur at the same time upon their simultaneous induction. Our analysis took advantage of the fact that the morphological events accompanying macropexophagy and microautophagy differ significantly, allowing an easy discrimination of the nature of the degradation events that take place. In short, macropexophagy is morphologically characterized by the sequestration of individual peroxisomes by multiple membrane layers that precede the fusion process to the vacuole to facilitate degradation by vacuolar hydrolases [6,7]. Microautophagy on the other hand does not require organelle sequestration but is effectuated via uptake of a portion of cytoplasm, including organelles, in a phagocytosis-like manner [5].

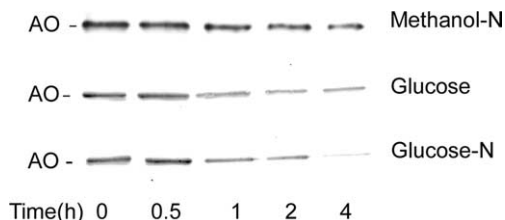


Fig. 1. Western blot experiments to show the kinetics of AO turnover at different autophagy conditions. Western blots were prepared from samples taken at the indicated time points of methanol-grown cells grown to the mid-exponential growth stage ($\text{OD}_{660} = 1.5$) and exposed to (i) methanol media that lack any nitrogen (N) source, (ii) 0.5% glucose and (iii) N-limitation and excess glucose simultaneously. Equal volumes of cultures were loaded per lane. The blots, decorated with α -AO antibodies show that the reduction of AO protein is enhanced at conditions of concurrent excess glucose and N-limitation conditions, relative to exposure of cells at either of these conditions alone.

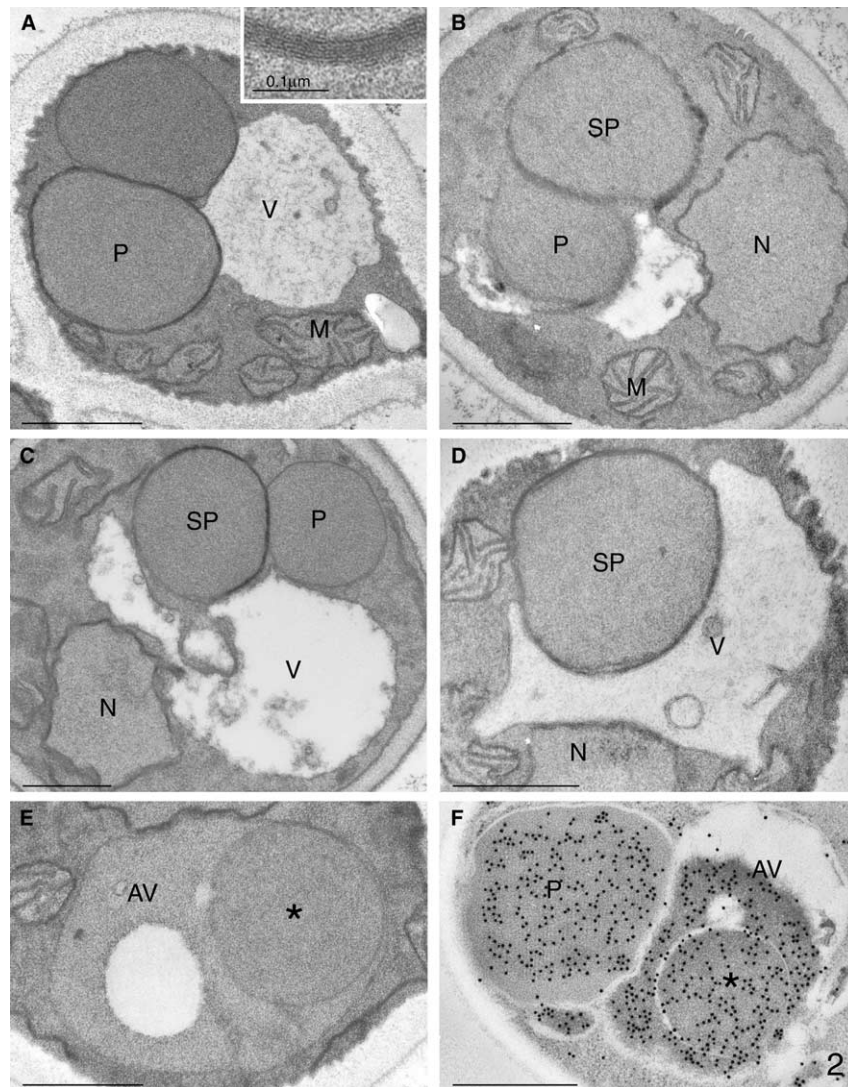


Fig. 2. (A) A sequestered peroxisome in a methanol-grown cell, shifted for 30 min to glucose. The inset shows a high magnification of the sequestering membrane layers. (B) A detail of a cell exposed for 30 min to glucose and N-limitation at the same time; shown is an organelle that is being, but not yet completely, sequestered and an adjacent one that is engulfed by a vacuole profile. (C) and (D) Two stages of the uptake of sequestered peroxisomes by vacuole engulfment that is typical for microautophagy. (E) and (F) Examples of the uptake of peroxisomes in autophagic vacuoles, showing the morphology (E) and immuno cytochemical demonstration of AO protein in the incorporated peroxisomes (*) as well as the autophagic vacuole (glutaraldehyde- α -AO antibodies-GAR gold). Electron micrographs are taken from KMnO_4 -fixed *H. polymorpha* cells unless otherwise stated. Abbreviations: AV, autophagic vacuole; M, mitochondrion; N, nucleus; P, peroxisome; SP, sequestered peroxisome; V, vacuole. The marker represents 0.5 μm (unless otherwise stated).

In the related methylotrophic yeast, *P. pastoris*, the mode of selective peroxisome degradation is dependent on the carbon source added. Excess glucose induces engulfment of clusters of peroxisomes, a process designated micropexophagy, while ethanol leads to removal of individual peroxisomes by a mechanism similar to that described above for *H. polymorpha* [12–14,24]. In baker's yeast, selective peroxisome degradation has been studied in cells pre-grown on oleic acid to induce peroxisome proliferation that was subsequently transferred to excess glucose conditions in the absence of a nitrogen source [15].

The above observations on *H. polymorpha* and *P. pastoris* suggest that different stimuli can induce at least two morphologically distinct mechanisms of peroxisome turnover. This raises the question which of these is operative in organelle degradation in baker's yeast cells when these are exposed to

excess glucose and nitrogen limitation simultaneously (cf. [15]). Hutchins et al. [15] concluded, based on their findings that the removal of various cytoplasmic marker proteins – Pho8 Δ 60p as cytosolic marker, F $_1$ β as mitochondrial marker and the Golgi marker Kex2p – occurred at a slower rate relative to the peroxisomal marker protein Fox3p, that peroxisome degradation in *S. cerevisiae* was a specific process. Our results, however, show that in *H. polymorpha* concurrent excess glucose and nitrogen limitation conditions speed up peroxisomal degradation because macropexophagy and microautophagy occur simultaneously. These results are consistent with the possibility that also in *S. cerevisiae*, the enhanced level of Fox3p degradation may actually be the result of the combined action of these two distinct autophagy-like processes, rather than exclusively a specific uptake of microbodies.

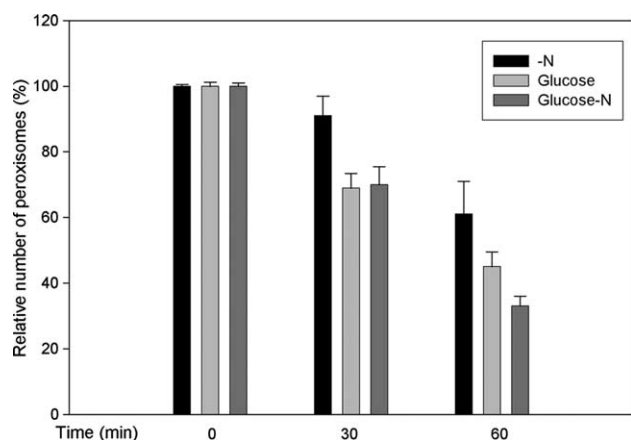


Fig. 3. Relative peroxisome numbers in cells subjected to various autophagy conditions. The average number of peroxisomes per cell was estimated by random counting of cell profiles in ultrathin sections of KMnO_4 -fixed cells. For growth conditions see legend to Fig. 1. Error bars denote standard errors (S.E.; $N = 115$). The data show that the relative reduction of peroxisome numbers is significantly enhanced in cells exposed to excess glucose and N-limitation simultaneously relative to N-limitation and excess glucose conditions alone. These differences (>3 S.E. difference) become prominent after 1 h of cultivation in the new environment.

It has been demonstrated in a number of yeast species that the various protein sorting pathways to the vacuole (e.g., autophagy, the Cvt pathway, pexophagy, endocytosis) share the function of various proteins [10,25–27]. However, recent studies have indicated that many of these proteins are not required for all processes, and that some are unique for a single process, in particular those that specify cargo selection such as Atg19¹ [28], Pex14 [29], Pex3 [30], Atg11 [31,32] and Atg23 [17]. Clearly, this challenges the approach that selective peroxisome degradation can be studied in *S. cerevisiae* using two different stimuli – excess glucose together with nitrogen depletion – simultaneously. This argument is stressed by the finding that in specific cases absence of one peroxisome degradation pathway actually induces the alternative pathway [4,33].

In conclusion, our data clearly indicate that two different autophagy-related pathways can proceed at the same time in *H. polymorpha*. If this property is also valid for other yeast species, this may severely hamper the analyses of the specific function of a single gene product in one particular process. This argues to search for conditions that specifically block either of the two pathways to allow in depth studies on these pathways separately.

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¹ Recently a new unified nomenclature for proteins involved in autophagy and related proteins was adopted, see [34].